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Award Number: DAMD17-01-1-0247

TITLE: Downstream Signaling Mechanism Underlying MAPK-Induced Generation of the ER-Negative Phenotype

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REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual Summary (1 Jul 2001 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Downstream Signaling Mechanism Underlying MAPK-Induced Generation of the ER-Negative Phenotype

5. FUNDING NUMBERS
DAMD17-01-1-0247

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Estrogen receptor α (ERα) negative breast tumors often overexpress growth factor receptors, resulting in increased growth factor signaling and hyperactivation of MAPK (ERK1 and ERK2). We have previously shown that ERα-positive MCF-7 cells engineered to stably overexpress various signaling molecules leading to MAPK hyperactivation lose expression of ERα without inducing its transcriptional activation. The downregulation of ERα in these cells is transcriptional and is a specific action of MAPK hyperactivation that is reversible by MAPK abrogation. Here, we show that downregulation of ERα is not mediated specifically by either ERK-1 or –2. TAM67, a construct preventing AP-1 transcriptional activity, was used to determine that AP-1 activity does not play a role in ER downregulation. AP-1 activity is upregulated in response to MAPK activation, and increased AP-1 activity has been observed in ERα negative and hormone independent breast cancers. However, these are the first data indicating mechanistically that despite data correlating increased AP-1 activity with hormone independence/ERα-negativity, increased AP-1 activity is not responsible for ERα downregulation. Use of a dominant negative RSK1 construct indicates that RSK1 activity does not downregulate ERα. Transfection of ERK2Δ19-25, which is dominant negative for nuclear MAPK substrates while allowing activation of cytoplasmic substrates, revealed that a cytoplasmic substrate of MAPK is responsible for the generation of the ERα-negative phenotype in these cells. Collectively, these data reveal that the association between increased AP-1 activity and the ERα-negative phenotype is correlative, not causative, and that a cytoplasmic MAPK substrate other than RSK1 is responsible for ERα downregulation in our cell line models.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Signal transduction, MAPK, AP-1, ERα, tamoxifen resistance	27
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Upon diagnosis, breast cancer is described as either estrogen receptor (ER)-positive or ER-negative. Patients with ER-positive tumors have a longer disease free and overall survival, and they respond better to hormonal therapies such as tamoxifen, which is easier to tolerate than cytotoxic chemotherapy [1]. Conversely, patients with ER-negative tumors tend to have more aggressive disease and must be relegated to much harsher chemotherapy regimens [2;3]. Unlike ER-positive tumors, ER-negative tumors tend to overexpress growth factor receptors such as EGFR and c-erbB-2, and they have been shown to have high levels of activation of downstream signaling molecules such as MAPK [4-6]. Previous studies indicated that the hyperactivation of MAPK is directly responsible for the downregulation of ER in breast cancer cells, and that this downregulation is reversible via abrogation of MAPK activity [7]. Consequently, the present study seeks to identify the mechanism of this MAPK induced phenomenon. The outcome of this study has the potential to impact the lives of breast cancer patients who may be able to benefit from a treatment protocol where the blocking of growth factor signaling through MAPK can return ER expression and tamoxifen sensitivity, allowing ER-negative patients to avoid the harsh side effects of cytotoxic chemotherapy.

Body

Statement of Work

Task 1. Identify whether MAPK-induced downregulation of ERα is mediated specifically by ERK1 or ERK2. (months 1-8)

- *Overexpress ERK1 or ERK2 using activated, wild type ERK constructs*
- *Abrogate ERK1 and ERK2 mediated signaling via dominant negative ERK1 and ERK2 constructs*

Addressed in appended manuscript.

Task 2. Identify the role of AP-1 and its composition in ERα downregulation. (months 6-18)

- *Determine AP-1 composition in ERα-negative and ERα+ cell lines using fos and jun family member-specific antibodies by Western blotting and antibody supershifting*

Pending final results of the second part outlined in Task 2, this section has not been completed. As preliminary data indicate that abrogation of AP-1 activity does not play a role in the downregulation of ER in these model cell lines, this set of experiments may be omitted. However, Santa Cruz makes a series of antibodies against all fos and jun family members that can be easily obtained should the need arise.

- *Abrogate AP-1 expression using a dominant negative jun construct, Tam67*

Addressed in appended manuscript.

Task 3. Assess the role of cytoplasmic substrates of MAPK in ERα repression. (months 18-36)

- *Determine the localization of the key MAPK substrate*

- *Compare pp90^{RSK} activity levels in ERα-negative and ERα+ cell lines using ant anti-phospho-pp90^{RSK} antibody*

Addressed in appended manuscript.

- *Generation of pp90^{RSK} constructs*

A constitutively active RSK construct has been obtained from Dr. Jeffrey Smith, and a dominant negative RSK construct has been obtained from Dr. John Blenis, so the construction of any constructs will not be necessary for the completion of this task.

- *Determine if pp90^{RSK} overexpression causes ERα downregulation in ERα+ cell lines*

Addressed in appended manuscript.

- *Determine if AIB1 (activated in breast cancer-1) plays a role in ERα downregulation in ERα+ cell lines*

Several factors indicated that AIB1 may be a worthwhile target for investigation. In an *in vitro* setting, AIB1 can be phosphorylated by MAPK {684}. In addition, nuclear AIB1 has been shown to have a higher migration when run on a gel next to AIB1 extracted from the cytoplasm {1265}. These data suggest that nuclear AIB1 is phosphorylated, or at least phosphorylated to a greater extent than cytoplasmic AIB1. Taken together, the possibility exists that AIB1 is a cytoplasmic substrate of MAPK. Perhaps AIB1, activated by MAPK phosphorylation, could coactivate the transcription of a repressor of ER transcription, thereby contributing to its downregulation. In addition, AIB1 itself can potentiate NFκB mediated transcription (246). These data suggest another intriguing scenario in which AIB1 could regulate ER expression. Phosphorylation by MAPK could lead to an increase in active AIB1, which could then coactivate transcription from a promoter containing an NFκB site, and this increase in transcription may result in ER downregulation. If the proposed model is correct, then AIB1 is a necessary component for the repression of ER, and loss of AIB1 would reverse MAPK-induced ER downregulation. MCF7 cells were transfected with siRNA targeted against AIB1 (a 21-mer corresponding to nucleotides 564-583 of the AIB1 coding region) and an ERE-containing or control (NON) luciferase reporter construct. In addition, the cells were cotransfected with the Δraf construct or an empty vector. Previous studies showed a consistent downregulation of ER activity in MCF7 cells transfected with the Δraf construct {739}, and similar results were obtained when cotransfecting control siRNA (figure 1), validating this technique. Figure 1 reveals that there is no difference in estrogen induction of ERE-luciferase in Δraf containing cells in the presence or absence of AIB1, suggesting that activation of AIB1 does not lead to the downregulation of ER. Interestingly, however, the depletion of AIB1 alone results in a decrease in ERE-luciferase that is similar to the decrease seen with Δraf transfection. In addition, Δraf transfection in the absence of AIB1 does not further downregulate ER. These data suggest two possibilities: AIB1 is necessary to coactivate the ER in MCF7 cells, and in its absence fully functional ER can not result in the transcription of ER target genes; or that signaling downstream of raf leads to a decrease in AIB1 protein or activity, and this contributes to ER downregulation.

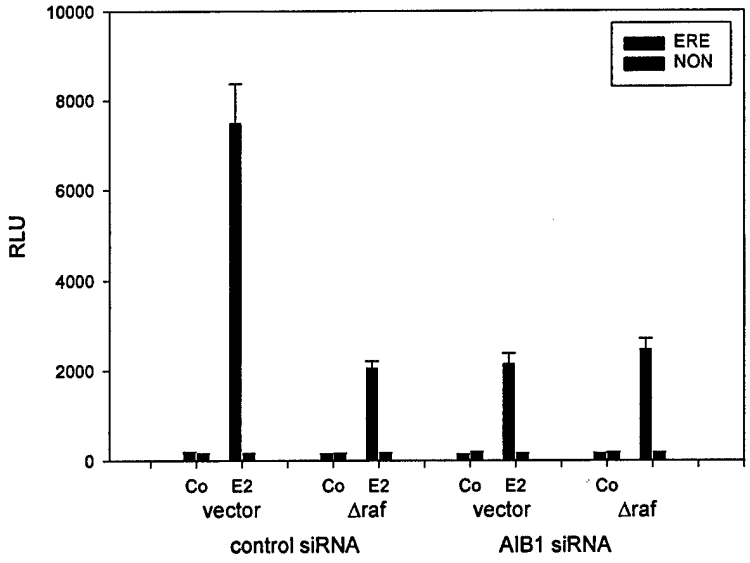

Figure 1. AIB1 depletion results in a decrease in ER activity. MCF7 cells were transfected with 1.25μg Δraf or vector control and 0.75μg ERE-luciferase or NON-luciferase as before. In addition, cells were simultaneously transfected with 6μL AIB1 or control siRNA. After transfection, the cells were treated with control or estrogen containing medium as indicated and harvested at 48 hours. This is a representative figure of two experiments, each done in triplicate, and the error bars represent s.e.m.

Depletion of AIB1 leads to a decrease in ER protein expression in MCF7 cells. If the downregulation of ER activity seen in the absence of AIB1 is a result of the estrogen receptor's need for AIB1 to fully activate transcription, then the decrease in ER activity should not be correlated with a decrease in ER protein. If, however, there is a decrease in ER protein, then AIB1 may be involved in ER downregulation as suggested in the proposed model above. In fact, ER protein is decreased in MCF7 cells with depleted AIB1; reinforcing the hypothesis that AIB1 may be involved in the downregulation of ER in cells with high MAPK. Densitometric scanning of a western blot of siRNA transfected MCF7 cells reveals that protein expression of AIB1 siRNA reduces ER levels to approximately one-third the levels seen in control siRNA treated cells (Fig. 2). MDA-231 cells are shown as an ER-negative control.

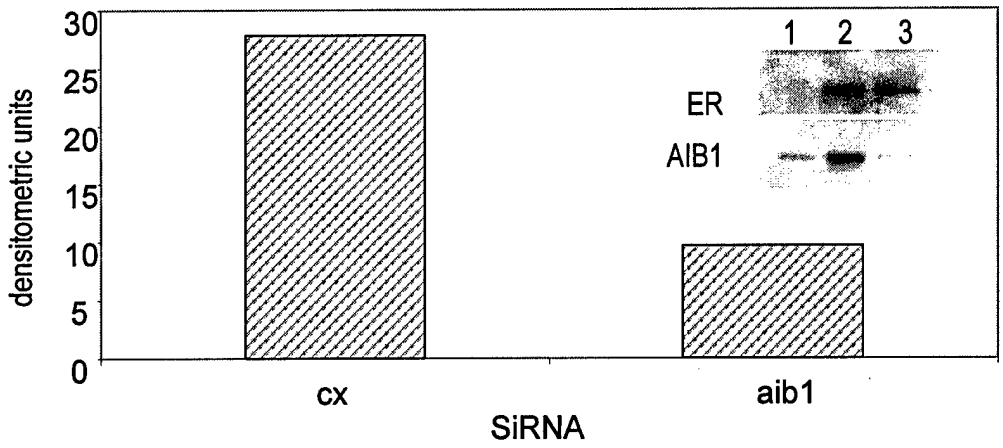

Figure 2. AIB1 depletion results in a decrease in ER protein. MCF7 cells were treated with control (lane 2) or AIB1 (lane 3) siRNA. Cells were harvested at 48 hours, and AIB1 and ER protein levels were assessed by western blotting 30μg of whole cell lysates. MDA-231 cells (lane 1) were also included in western blotting as an ER-negative control. Graph represents a densitometric analysis of a representative blot (shown in inset) of two separate experiments.

ER loss and MAPK hyperactivation result in an increase in AIB1 expression. The data thus far seem to correspond with the second proposed model, which is that MAPK activity somehow decreases AIB1 activity or expression, leading to ER downregulation. In this case, cells with MAPK-induced downregulation would be expected to have decreased levels of AIB1 expression or activity. However, both Raf 14c cells and MB3 cells show an increase in AIB1 expression compared to MCF7 cells (Fig 3). Attempts to overexpress Δraf in MCF7 cells in a transient setting were unfruitful, as the transfection efficiency of MCF7 cells is too low to see any impact of Δraf transfection on AIB1 expression (data not shown). While it is unlikely that an increase in AIB1 expression is correlated with a decrease in activity, it is at present a very difficult question to address. Currently, the only method for the assessment of AIB1 activity is through the use of reporter constructs containing hormone response elements. However, in this project, it is necessary to assess AIB1 activity independent of any estrogen regulated events, and therefore this analysis cannot be undertaken. Perhaps in the future, the activating phosphorylation sites on AIB1 will be identified, and the development of phospho-specific antibodies will enable the assessment of AIB1 activity independent of an effect on hormone responsive promoters.

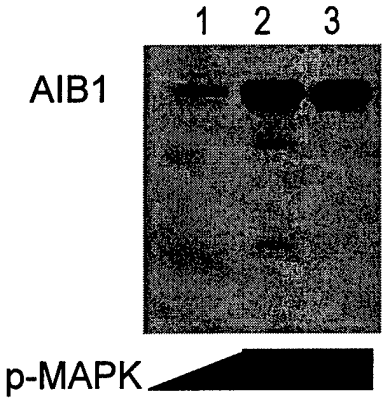

Figure 3. Loss of ER correlates with increased AIB1 expression. MCF7 (lane 1), Raf14c (lane 2), and MB3 (lane 3) cells were grown to approximately 80% confluence. Whole cell lysates were prepared, and western blotting was performed on 30μg of each sample. Relative amount of phospho-MAPK are indicated below the figure. This is a representative figure of two experiments.

Like the experiments investigating the role of RSK and AP-1 in ER downregulation, these data indicate that AIB1, as well, does not play a role in MAPK-induced downregulation of ER. Separate pieces of data indicate a role for AIB1 in ER regulation; with AIB1 resulting in the transcription of an ER repressor to downregulate ER (as evidenced by the upregulation of AIB1 in the ER-negative cell lines), or with AIB1 enhancing ER transcription, either directly or through the transcription of a gene coding for a factor which enhances ER transcription (as evidenced by the downregulation of ER protein with AIB1 depletion). However, the data as a whole do not support either conclusion. The fact that AIB1 depletion results in a decrease in ER protein (Fig. 2) is the most compelling data, implicating AIB1 in ER downregulation. However, the summation of the data suggests that any ER downregulation is independent of the ER repression reversed by the abrogation of MAPK signaling.

Key Research Accomplishments

- Determination that the abrogation of either ERK1 or ERK2 or both ERKs in combination leads to the reversal of ER downregulation
- Determination that abrogation of AP-1 mediated transcription does not reverse ER downregulation in ER-negative model cell lines
- Determination that a cytoplasmic substrate of MAPK other than pp90RSK is responsible for the downregulation of ER
- Determination that AIB1 is not responsible for the MAPK-induced downregulation of ER

Reportable Outcomes

Abstracts

Murthy, S., **Holloway, J.N.,** and El-Ashry D. 2004 A cytoplasmic substrate of mitogen activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: the role of nuclear factor-kappaB. Mol Endocrinol 18:1396-1410

Murthy, S., **Holloway, J.N.,** and El-Ashry D. Identification of MAP kinase substrates responsible for the downregulation of ERα in breast cancer cells. 94th Annual Meeting of the American Association for Cancer Research, Washington, DC, 2003, Abstract #1953

Holloway, J.N., Alexander, J., and El-Ashry, D. A Substrate of MAPK is Responsible for the Downregulation of ERα in Breast Cancer Cells. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, 2002. Abstract # 5332.

Conclusions

Previous data indicated that hyperactivation of MAPK results in the downregulation of ER in ER-positive breast cancer cells, and that this downregulation is reversible through the abrogation of both ERK1 and ERK2, either through MEK inhibition with U0126, or through the use of dominant negative constructs. We have now demonstrated that this ER downregulation is not a result of a specific substrate of either ERK1 or ERK2, as abrogation of either ERK or a combination of the two will result in the return of ER in ER-negative cells. As AP-1 family members are key MAPK substrates, we examined the effect of AP-1 abrogation on ER-negative cell lines to determine if clinical data correlating high AP-1 activity with ER-negativity had a causative relationship. Our data indicate that high AP-1 activity does not result in the downregulation of ER in our model cell lines, and this is the first data demonstrating that while there is significant clinical data correlating ER-negativity with high AP-1 activity, this AP-1 activity is not responsible for the downregulation of ER and acquisition of hormone independence. Experiments with the ERK2Δ19-25 construct revealed that the substrate of MAPK responsible for the downregulation of ER resides in the cytoplasm. In addition, use of the dominant negative RSK construct provided data indicating that RSK is not the responsible cytoplasmic substrate. Unfortunately, like AP-1 and RSK, AIB1 is not the MAPK substrate responsible for the MAPK-induced downregulation of ER. Determining the identity of the

MAPK substrate that is responsible for ER downregulation may enable ER-negative patients to be treated with an inhibitor of that specific molecule, returning ER expression and tamoxifen sensitivity, allowing them to be treated with hormonal therapy and forgo the side effects that accompany cytotoxic chemotherapy.

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Appendices

"Murthy, S., **Holloway, J.N.,** and El-Ashry D. 2004 A cytoplasmic substrate of mitogen activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: the role of nuclear factor-kappaB. Mol Endocrinol 18:1396-1410" is appended as file "appendix1.pdf"

0888-8809/04/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 18(6):1396–1410

doi: 10.1210/me.2004-0048

A Cytoplasmic Substrate of Mitogen-Activated Protein Kinase Is Responsible for Estrogen Receptor-α Down-Regulation in Breast Cancer Cells: The Role of Nuclear Factor-κB

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Estrogen receptor α (ERα) negative breast tumors often present with enhanced expression and/or activation of growth factor receptors, resulting in increased growth factor signaling and hyperactivation of MAPK (ERK1 and ERK2). We have previously shown that ERα(+) MCF-7 cells with elevated growth factor signaling lose expression of ERα without any ligand-independent transcriptional activation, and this is a reversible effect attributable to ERK1/2 hyperactivation. Here, we show that down-regulation of ERα is not mediated by a specific ERK-1 *vs.* ERK-2 substrate. Despite up-regulated activator protein-1 activity in response to ERK1/2 activation, and in ERα(−) and hormone-independent breast cancers, we find that increased activator protein-1 activity is not responsible for ERα down-regulation. Interestingly, our findings implicate a cytoplasmic substrate of ERK1/2. However, RSK1, the best-characterized cytoplasmic ERK1/2 substrate, does not down-regulate ERα in our models. On the other hand, inhibition of nuclear factor-κB (which is linked to chemoresistance in cancer in general and has elevated activity in hormone-independent and ERα− breast cancer) significantly enhances ERα activity, suggesting that indirect elevation in nuclear factor-κB activity (due to hyperactive ERK1/2) is at least partially responsible for ERα down-regulation in these cell line models. (*Molecular Endocrinology* 18: 1396–1410, 2004)

BREAST CANCER CAN present as estrogen receptor α (ERα) positive (+) or negative (−). The presence or absence of ERα is a key prognostic feature of this disease: ERα+ tumors have a better prognosis and respond to hormonal therapy (1), whereas ERα− tumors have overall a worse prognosis and are resistant to hormonal therapy (2, 3). ERα+ tumors can progress over time and after antiestrogen therapy to ERα− tumors. For example, in patients with ERα+ primary tumors who relapse after adjuvant tamoxifen therapy, 50% of the recurrent tumors lack ERα expression. About a third of metastatic tumors that initially respond to tamoxifen subsequently develop resistance and lose ERα expression over this period of time (4, 5). Progression to an ERα− phenotype typically involves the constitutive overexpression of growth promoting genes that are normally regulated by estrogen, thereby leading to a loss of estrogen dependence, resistance to antiestrogens, and a more aggressive phenotype overall. It is also possible that ERα− tumors develop *de novo*. Immunohistochemical studies of normal breast tissue suggested that approximately 6–12% of the ductal epithelial cells are ERα+ (6, 7), although some studies with more sensitive antibodies indicate that the actual number is higher (8). Interestingly, the proliferating ductal epithelial cells in the normal breast do not express ERα (6, 8–11), suggesting that ERα− cells give rise to ERα− tumors. Whether the ERα− phenotype is acquired or *de novo*, the lack of ERα expression denotes a more aggressive phenotype as well as resistance to antiestrogens, and as a result, precludes the use of tamoxifen, relegating patients to more toxic chemotherapies (2, 3).

ERα− tumors and cell lines often overexpress certain growth factor receptors such as the EGFR [epidermal growth factor (EGF) receptor] and c-erbB-2. EGFR and c-erbB-2 overexpression are also important prognostic indicators in breast cancer, independent of their inverse correlation with ERα expression (12–17). This increased growth factor receptor expression and/or activation correlates with increased MAPK activity, both in tumors and in cell lines (12, 18–20). In addition to overexpression and/or constitutive activation of growth factor receptors (with the resulting hyperactivation of MAPK), many hormone-independent

Abbreviations: AP, Activator protein; CCS, charcoal-stripped calf serum; CMV, cytomegalovirus; co-MCF-7, control transfected MCF-7; DMSO, dimethylsulfoxide; dnERK, dominant-negative ERK; dnSK1, dominant-negative RSK1; EGF, epidermal growth factor; EGFR, EGF receptor; ERα, estrogen receptor-α; ERE, estrogen response element; HB-EGF, heparin-binding EGF; IκB, inhibitor of κ-B; IKK, IκB kinase; IMEM, improved MEM; luc, luciferase; MMTV, mouse mammary tumor virus; NF-κB, nuclear factor-κB.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

and ERα– tumors also have elevated activator protein (AP)-1 activity (21, 22). Elevated activity of AP-1 and related family members has also been implicated in breast tumor progression in xenograft models: c-jun overexpression in MCF-7 cells results in a hormone-resistant, tumorigenic phenotype (23).

Another transcription factor that might be linked to hormone-independent breast cancer, as well as elevated growth factor signaling, is nuclear factor-κB (NF-κB). NF-κB activity is elevated in hormone-independent breast cancer (24), and is implicated in enhanced cell survival and chemoresistance in cancer (25–27). NF-κB exists in the cytoplasm in the form of a complex with IκB (inhibitor of κ-B). Cytokines, chemokines, and intracellular stress lead to the phosphorylation of IκB by IKK (IκB kinase), releasing NF-κB, which can then translocate into the nucleus and modulate the transcription of target genes (reviewed in Ref. 28). Although MAPK does not directly phosphorylate IκB or activate IKK, there is evidence to show that hyperactivation of MAPK leads to elevated NF-κB-mediated transcriptional activity through induction of an autocrine factor, most likely heparin-binding EGF (HB-EGF) (29, 30). These data suggest that our model cell lines (with elevated growth factor signaling pathways) might have elevated NF-κB activity and that this might be involved in the interactions between up-regulated growth factor signaling and decreased ERα expression.

We have previously used stable overexpression of various growth factor signaling components in ERα+ MCF-7 breast cancer cells to study the interaction between ERα signaling and growth factor receptor signaling in breast cancer (31–34). Using cell lines that overexpress constitutively active forms of Raf-1, MEK1, or c-erbB-2 and ligand-activatable EGFR, we have shown that the resultant hyperactivation of MAPK (ERK1/2) activity through these signaling pathways leads to the down-regulation of ERα. We have further shown that this down-regulation is not a consequence of ligand-independent ERα activation, and that it is reversible *in vitro*. Abrogation of ERK activity using either pharmacologic inhibitors or dominant-negative ERK (dnERK) constructs reverses the ERα down-regulation and restores ERα activity (34). The objective of this study is to further elucidate the mechanisms by which elevated ERK signaling leads to ERα down-regulation. First, using dnERK1 and dnERK2, we show that the ERK substrate(s) responsible for ERα down-regulation is a common ERK1/ERK2 substrate and not specific to either ERK isoform. Next, the potential role of two key ERK substrates, AP-1 and RSK1, in ERα down-regulation was analyzed. Overexpression and increased activation of AP-1, a transcription factor, has been correlated with hormone independence and the ERα-negative phenotype (21, 35). RSK is a kinase responsible for the activation of various transcription factors (including ERα) and is also involved directly in chromatin remodeling (reviewed in Ref. 36). Although our hyperactive MAPK cell lines have significantly increased AP-1 activity relative to parental MCF-7 cells, inhibition of this AP-1 activity using a dominant-negative jun does not reverse the ERα down-regulation. Similarly, inhibition of increased RSK activity using a dominant-negative RSK1 (dnRSK1) does not restore ERα expression. Instead, dnRSK1 reduces ligand-induced ERα activation in our control cells, further supporting the possibility that phosphorylation of ERα by RSK enhances ERα transactivation (37). Interestingly, studies with an ERK2 deletion construct that selectively abrogates nuclear ERK1/2 activities show that the ERK substrate responsible for ERα down-regulation is located in the cytoplasm. Finally, we show that these cell lines have elevated NF-κB activity compared with parental MCF-7 cells, and this elevation in NF-κB activity is attributable to enhanced growth factor signaling through ERK1/2. Inhibiting this NF-κB activity either pharmacologically, or through the expression of a constitutively active IκB [(ca)IκB], partially restores ERα activity and expression in these cells. Our findings suggest a role for cytoplasmic substrates of MAPK in ERα down-regulation in breast cancer and further support a role for MAPK-induced NF-κB activity in this down-regulation.

RESULTS

ERα Down-Regulation Due to Hyperactive MAPK Is Not Specifically Attributable to ERK1 or 2

Previous work from our lab has shown that hyperactivation of MAPK results in the down-regulation of ERα protein and message and that this down-regulation is reversible through the abrogation of MAPK (ERK 1/2) signaling, either via pharmacologic inhibition or through expression of dnERK1 and dnERK2 (34). Although ERK1 and ERK2 share many substrates, they have some isoform-specific substrates as well (38). Therefore, our first step in the elucidation of a substrate responsible for ERα down-regulation in breast cancer cells was to determine whether the MAPK-induced down-regulation was a specific effect of the hyperactivation of either ERK1 or ERK2, or whether signaling through either MAPK would down-regulate ERα. Cell line models with drastically reduced ERα expression (described previously in Ref. 34) were obtained by the stable transfection and overexpression of various signal transduction factors into ERα(+) MCF-7 breast cancer cells. These signaling factors were a constitutively active c-Raf-1 [yielding Raf14c cells or (ca)Raf cells] (39), a constitutively active MEK-1 construct (40) [yielding MEK15c or (ca)MEK cells], a wild-type EGFR that can be activated by ligand [MCE5 or EGFR(+) cells] (32), or a wild-type c-erbB-2 [a clone with constitutively high levels of autophosphorylation and constitutive downstream signaling, MB3 or erbB2(+) cells] (33). All of these cell lines grow continuously in the absence of estrogen,

have very high levels of MAPK activity (consistent with the levels of MAPK activity found in ERα− breast cancer cell lines) and express extremely low levels of ERα when compared with control transfected MCF-7 cells (co-MCF7) (34). Each cell line expresses between 4 and 20 fmol of ERα/mg protein, a significant reduction when compared with the control transfected cell lines (Table 1), which exhibit about 120 fmol/mg protein when growing in the continuous presence of estrogen (co-MCF7) or about 400 fmol/mg protein when growing in the continuous absence of estrogen (co-MCF7/s). By selecting single clones of multiple cell lines, all with hyperactive MAPK but generated in different ways, we have substantially reduced the possibility that the reversible down-regulation of ERα seen in these lines is a chance effect due to clonal variation.

To determine whether the ERK substrate responsible for ERα down-regulation is specific to ERK-1 or ERK-2, cells were transiently cotransfected with either an empty vector, dnERK1 (41), dnERK2 (42), or both, in addition to a luciferase (luc) reporter construct (Fig. 1). The estrogen response element (ERE)-luc construct was derived as previously described by the removal of the glucocorticoid response element from the mouse mammary tumor virus (MMTV) promoter. It was replaced with two tandem EREs and then inserted upstream of the luciferase gene. The NON-luc reporter (a critical control that corrects for any nonspecific activation due to the remaining elements of the MMTV promoter) is identical with the ERE-luc, except that the ERE sequences have been scrambled to produce a nonsense sequence. We have previously shown, both by ligand binding assays and by immunohistochemistry, that transfection of a combination of dnERKs 1 and 2 is sufficient to return ERα expression, and through transient transfection assays with ERE-luc and NON-luc that this reexpressed ERα is functional. Importantly, we found that receptor expression, as measured by ligand binding assay or by immunohistochemistry, correlated very well with receptor function as measured by the transient transfection assays with ERE-luc (34), confirming that receptor activity mirrors receptor expression. In addition to assessing ER expression by ligand binding assay, many studies were performed using either immunohistochemistry or Western blotting (34). Because all of these latter studies used ERα-specific antibodies, and ERα expression measured in this way fully correlated with ER activity by ERE-luc assay, it is presumed that we are looking at ERα-specific effects. Furthermore, the dnERK constructs do not affect ERE-luc activity in the co-MCF7 cells, either in the absence or presence of estrogen (Fig. 1A), again indicating that modulation of ERK activity does not affect the level of activation of preexisting receptor. In all four cell line models with hyperactive MAPK [(ca)Raf in Fig. 1B, (ca)MEK in 1C, erbB2(+) in 1D, and ligand-activated EGFR(+) in 1E], expression of either dnERK is sufficient to restore ERα to levels comparable with the combination of dnERKs 1 and 2 that we previously described as able to return ERα expression. Although any one dnERK alone was perhaps a little less effective at restoring ERα function compared with the combination of both dnERKs, both dnERK-1 and dnERK-2 restored ERα function to levels that would be physiologically relevant. Parallel transfection of the NON-luc does not reveal nonspecific promoter activation, but rather that the ERα activity measured is a direct result of estrogen-induced ERα signaling. Because both dnERKs were sufficient to return ERα to comparable levels, the ERK substrate responsible for ERα down-regulation is unlikely to be specific to either one.

Table 1. ERα Expression in MCF-7-Derived Cells with Hyperactive MAPK

Cell Line	ERα Expression (fmol/mg protein)
(ca)Raf	6–10
(ca)MEK	4–7
erbB2(+)	20
EGFR(+) +EGF	6–8
co-MCF7	120
co-MCF7/s	400

ERα expression in our model cell lines was measured by ligand binding assay several times (minimum of three) over a wide range of passage number as described in *Materials and Methods*. The range of receptor levels from these multiple measurements are expressed as femtomoles per milligram of cellular protein.

Neither AP-1 nor RSK Are Responsible for ERα Down-Regulation Due to Hyperactive MAPK

AP-1 plays a prominent role in the transcriptional activation of many genes. Furthermore, AP-1 activity is more frequently elevated in ERα− breast cancer (21, 35, 43, 44), and c-jun overexpression confers a hormone-independent, ERα−, tumorigenic phenotype on MCF-7 cells in animal models (23). In addition, the expression of AP-1 family members such as fra-1, fra-2, and some jun family members are specifically induced by MAPK (45, 46). Thus, it would be expected that AP-1 activity would be elevated in systems with hyperactive MAPK. Therefore, to determine the role of elevated AP-1 activity in ERα down-regulation in these MCF-7 models with hyperactive MAPK, a dominant-negative jun construct (TAM67) (47) was used to inhibit AP-1 activity. AP-1 molecules consist of a dimer composed of a jun family member and either another jun family member or a fos family member. TAM67 acts as a dominant negative by forming inactive homo- and heterodimers with jun and fos family members in the cell, thereby eliminating AP-1 mediated transcriptional activity.

The first panel of Fig. 2 represents the transient cotransfection of TAM67 or its parental vector, pCDNA3, along with the AP-1-luc reporter in our model cell lines, as well as in control transfected MCF-7 cells (co-MCF7). All of these cell lines show

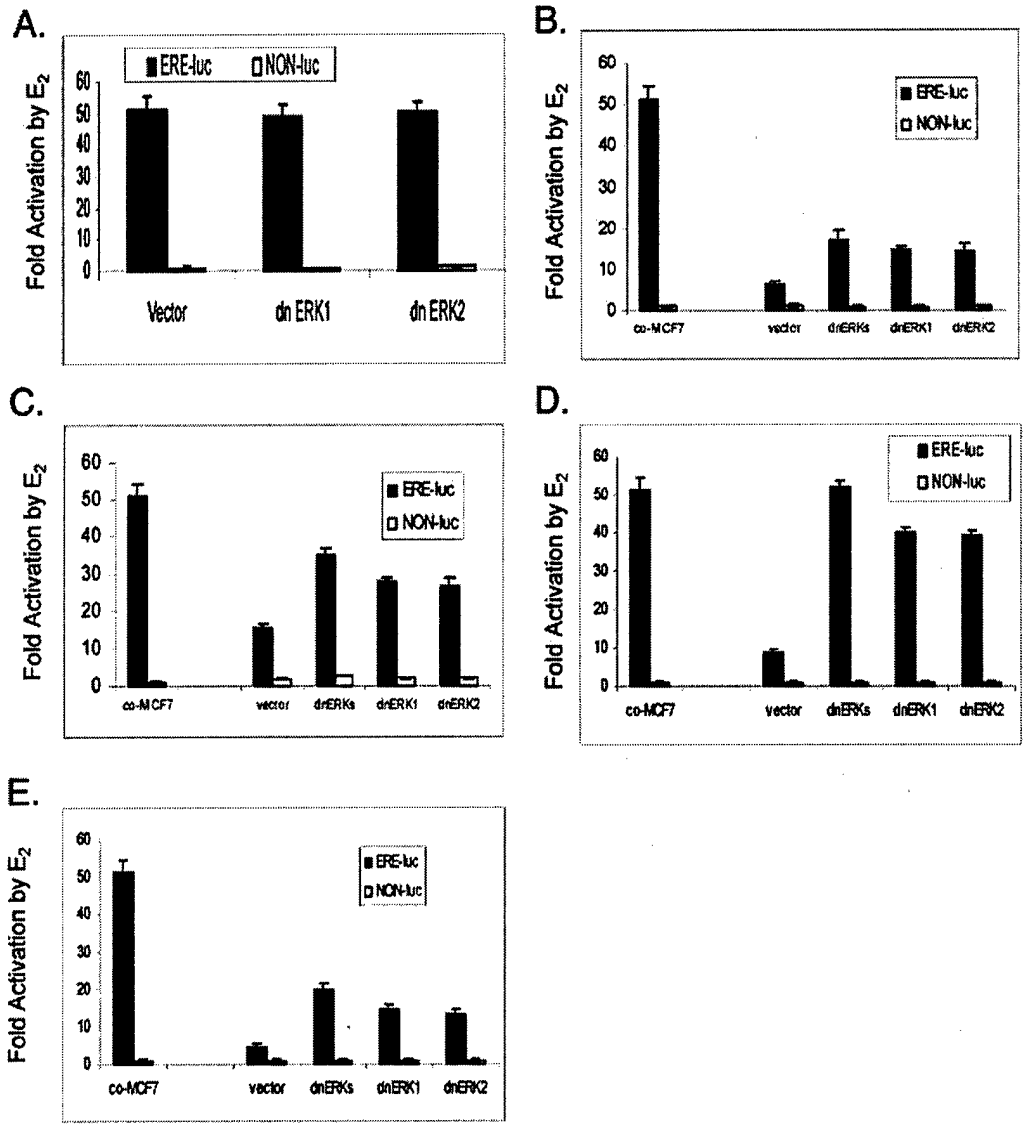

Fig. 1. Down-Regulation of ERα Is Not Mediated Exclusively by Either ERK1 or ERK2

A–E, Co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg total dnERK constructs (either dnERK1 alone, or dnERK2 alone, or a 1:1 combination of both dnERKs) and 0.75-μg luciferase reporter constructs and treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. The NON-luc construct is an identical plasmid, except that the EREs are scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each performed in triplicate. *Error bars* represent SEM.

elevated AP-1 activity (relative to co-MCF7), which is inhibited 50–75% by the TAM67 construct, reducing the AP-1 activity in these hyperactive MAPK cells to levels comparable to ERα+ co-MCF7 cells (Fig. 2A). If AP-1 were playing a central role in the down-regulation of ERα in these breast cancer cells, then the expression of TAM67 (and therefore the specific abrogation of AP-1 activity) in the low ERα model cells should result in the restoration of ERα activity and expression. However, transfection of TAM67 did not restore ERα levels to any significant extent in any of the cell lines (Fig. 2, B–E). In all of these cell lines, expression of TAM67 did not result in estrogen induction that was significantly different from the vector control, despite AP-1 activity being consistently reduced. Cotransfection of dnERKs, on the other hand, fully restored ERα function in all these cell lines. These data indicate that elevated AP-1 activity does not play a role, either directly or indirectly, in the down-regulation of ERα that results from the hyperactivation of MAPK.

RSK is a cytoplasmic substrate of MAPK that translocates to the nucleus upon activation, activates several transcription factors, and regulates chromatin

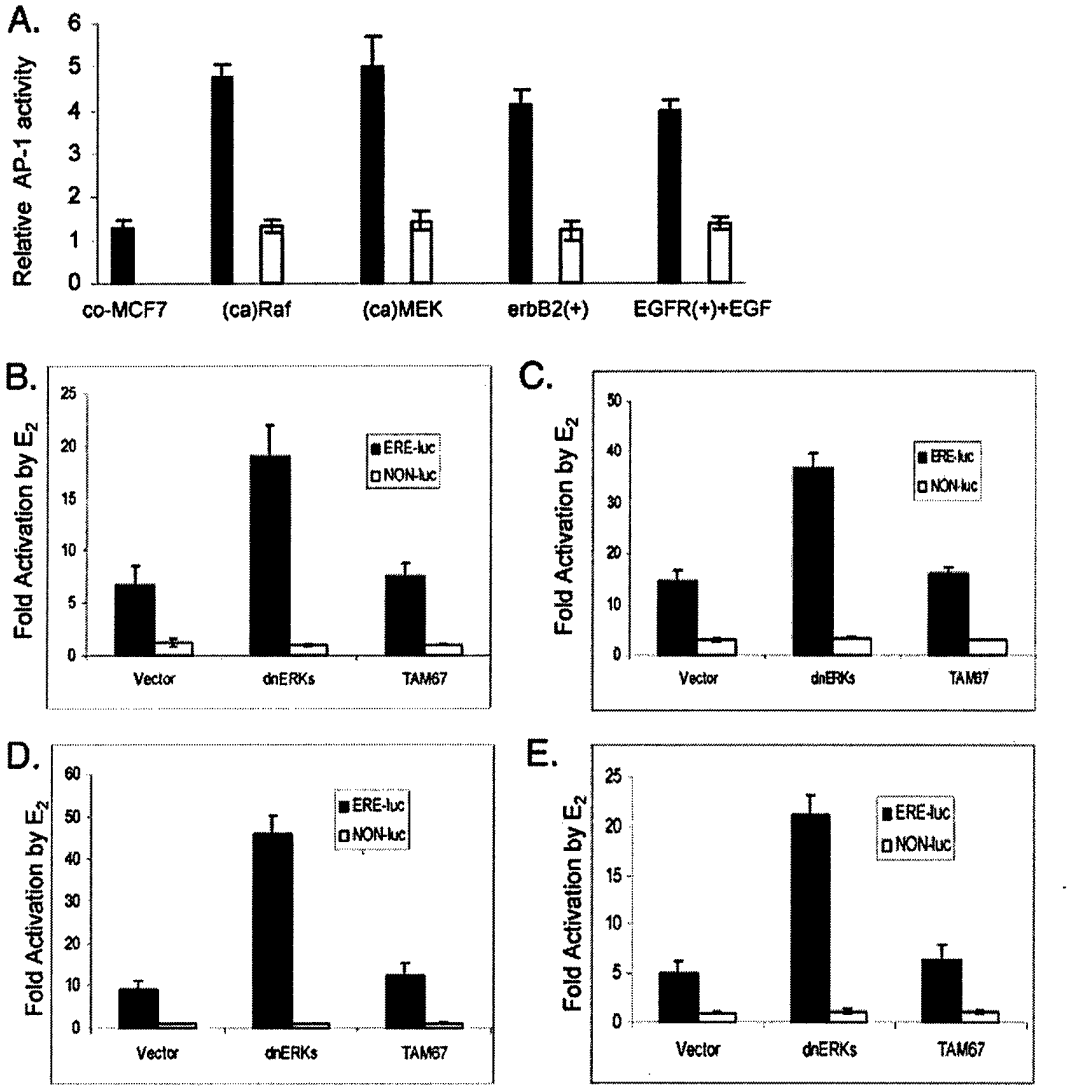

Fig. 2. Down-Regulation of ERα Is Not Mediated by AP-1 Activity

A, Expression of TAM67 inhibits AP-1 activity. Cells were transiently transfected with 1.25 μg TAM67 or vector control DNA and 0.75 μg AP-1-luc reporter construct, and basal AP-1 activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative AP-1 activity. The *black bars* represent basal AP-1-luc activity, whereas the *white bars* represent AP-1-luc activity upon cotransfection of the TAM67 plasmid. The *first bar* shows AP-1 activity in co-MCF7 cells. The next four pairs show AP-1 activity in (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells respectively. B–E, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg TAM67 (dnjun construct) and 0.75 μg ERE-luc or NON-luc reporter constructs and were treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each performed in triplicate. *Error bars* represent SEM.

structure through the phosphorylation of histones. Thus, increased activation of RSK1 could lead to induction of a factor that represses ERα expression. On the other hand, RSK1 has also been reported to phosphorylate ERα directly on serine 167, leading to ligand-independent activation of ERα (37). These data indicate that RSK1 might play a role in enhancing ERα activity, either in a ligand-dependent or ligand-independent fashion. In our model cell lines, RSK1 expression is unchanged in response to elevated MAPK activity, but its activation correlates with MAPK activity, and therefore is high in these model cell lines (Fig. 3A).

To determine the effects of elevated RSK1 activity on ERα in these model cell lines, a dnRSK1 construct was obtained (48). Because RSK1 had been reported to activate ERα, the effects of dnRSK1 on ERα activity in co-MCF7 cells was first examined. Indeed, cotransfection of dnRSK1 into ERα+ co-MCF7 cells results in reduced estrogen-induced transcriptional activation (Fig. 3B), indicating the dnRSK1 has inhibitory activity

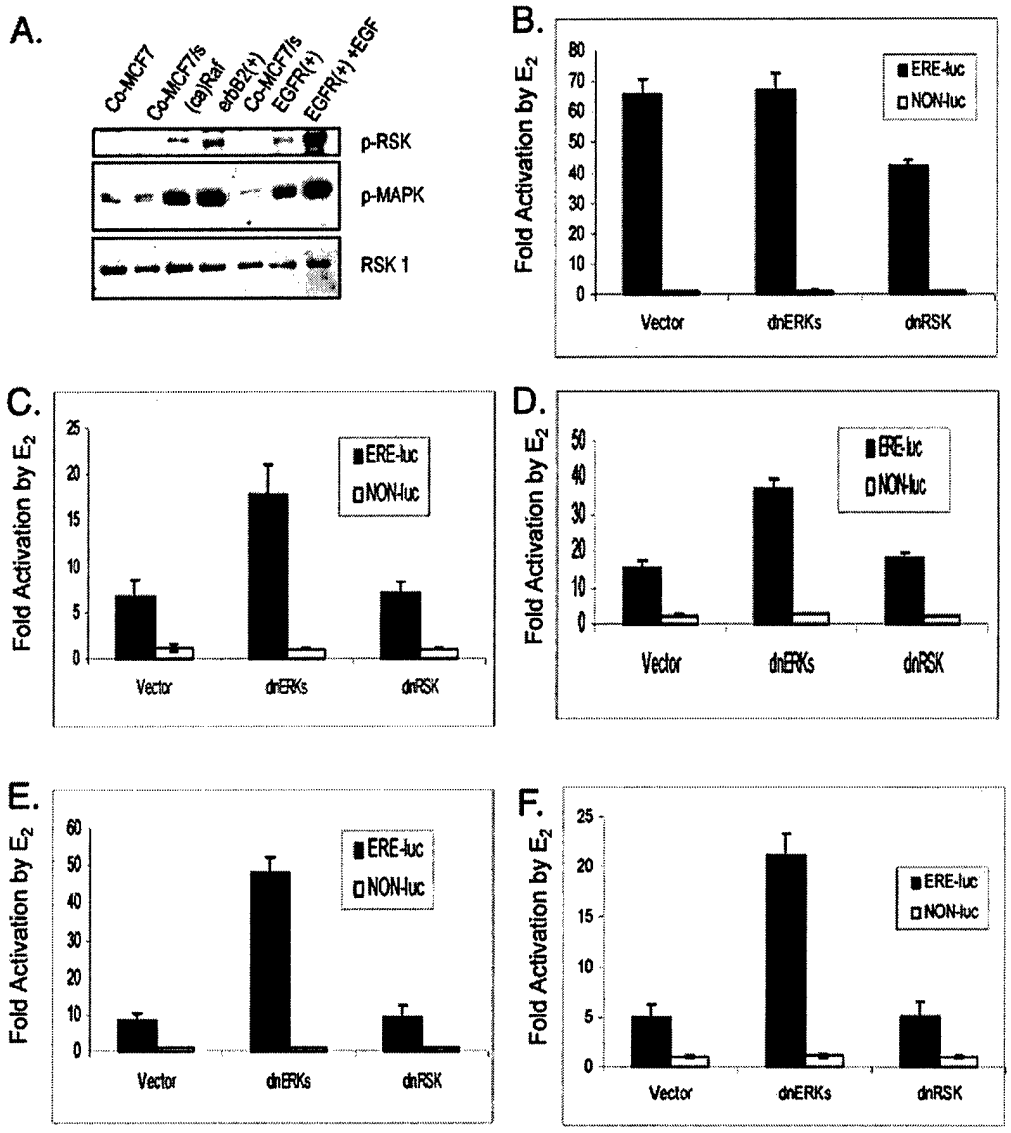

Fig. 3. Down-Regulation of ERα Is Not Mediated by RSK1

A, RSK1 activity correlates with MAPK activity in breast cancer cells. Whole cell lysates were prepared from cells in normal culture conditions grown to approximately 80% confluence. EGFR(+) cells were treated or not with 10 ng/ml EGF for 10 min. Western blots were performed on 5 μg of total protein for phospho-RSK1 and phospho-MAPK. A Western blot for RSK1 is shown as a loading control. B–F, co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg dnRSK1 construct and 0.75 μg luciferase reporter constructs and were treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each performed in triplicate. *Error bars* represent SEM.

against RSK1. If RSK1 hyperactivation were responsible for the down-regulation of ERα, then expression of dnRSK1 in the low ERα model cell lines would result in the restoration of ERα activity to significantly higher levels (comparable to those seen with dnERKs). However, transient transfection of dnRSK1 into our model cells was not able to restore ERα to levels comparable with dnERK transfection, indicating that RSK1 is not responsible for the MAPK-induced down-regulation of ERα (Fig. 3, C–F). In fact, in co-MCF-7 cells, the estrogen-inducible ERα activity even decreases slightly. These data collectively indicate that hyperactivation of RSK1 is not mediating the down-regulation of ERα induced by hyperactive MAPK.

A Cytoplasmic Substrate of MAPK Is Responsible for ERα Down-Regulation

MEK acts as a cytoplasmic anchor for MAPK, but upon MEK activation by Raf, MAPK is activated and released into the cytoplasm, where it can activate cytoplasmic substrates such as RSK1. In addition,

active MAPK translocates to the nucleus and phosphorylates nuclear substrates such as elk (reviewed in Ref. 49). A mutant ERK2 construct lacking the region that associates with MEK (ERK2Δ19–25) (50) was used to determine the role of cytoplasmic *vs.* nuclear MAPK activity in ERα down-regulation. Deletion of the MEK association region causes the construct to be constitutively localized in the nucleus, where it cannot be activated by MEK. As a result, this construct prevents any MAPK activity in the nucleus by binding to (and therefore blocking docking sites on) nuclear MAPK substrates. However, endogenous cellular MAPK can still be activated in the cytoplasm and can continue to activate cytoplasmic substrates. Essentially, this construct functions as a dominant negative in the nucleus without affecting cytoplasmic MAPK signaling (50). As would be expected, nuclear MAPK activity (mea-

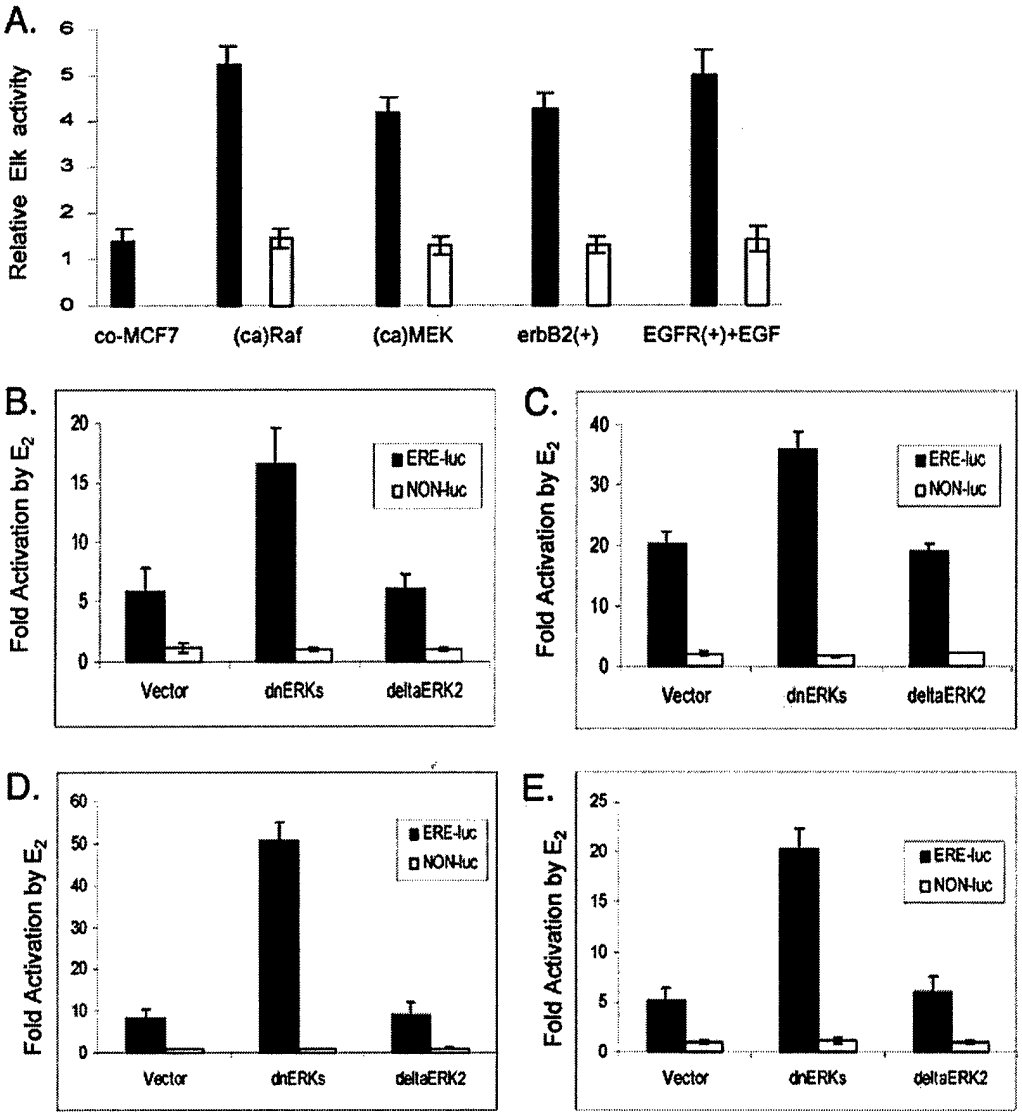

Fig. 4. Down-Regulation of ERα Is Mediated by a Cytoplasmic Substrate of MAPK

A, ERK2Δ19–25 represses activity of a nuclear MAPK substrate. Cells were transiently transfected with 0.625μg ERK2Δ19–25 or vector control DNA, 0.625 μg pFAelk, and 0.75 μg pFA-luc reporter construct, and basal Elk activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative Elk activity. The *black bars* represent basal Elk activity, whereas the *white bars* represent Elk activity upon cotransfection of the ERK2Δ19–25 plasmid. The *first bar* shows Elk activity in co-MCF-7 cells; the next four pairs show Elk activity in in (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells respectively. B–E, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg ERK2Δ19–25 construct and 0.75 μg luciferase reporter constructs and were treated with control or estrogen-containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each done in triplicate. *Error bars* represent SEM.

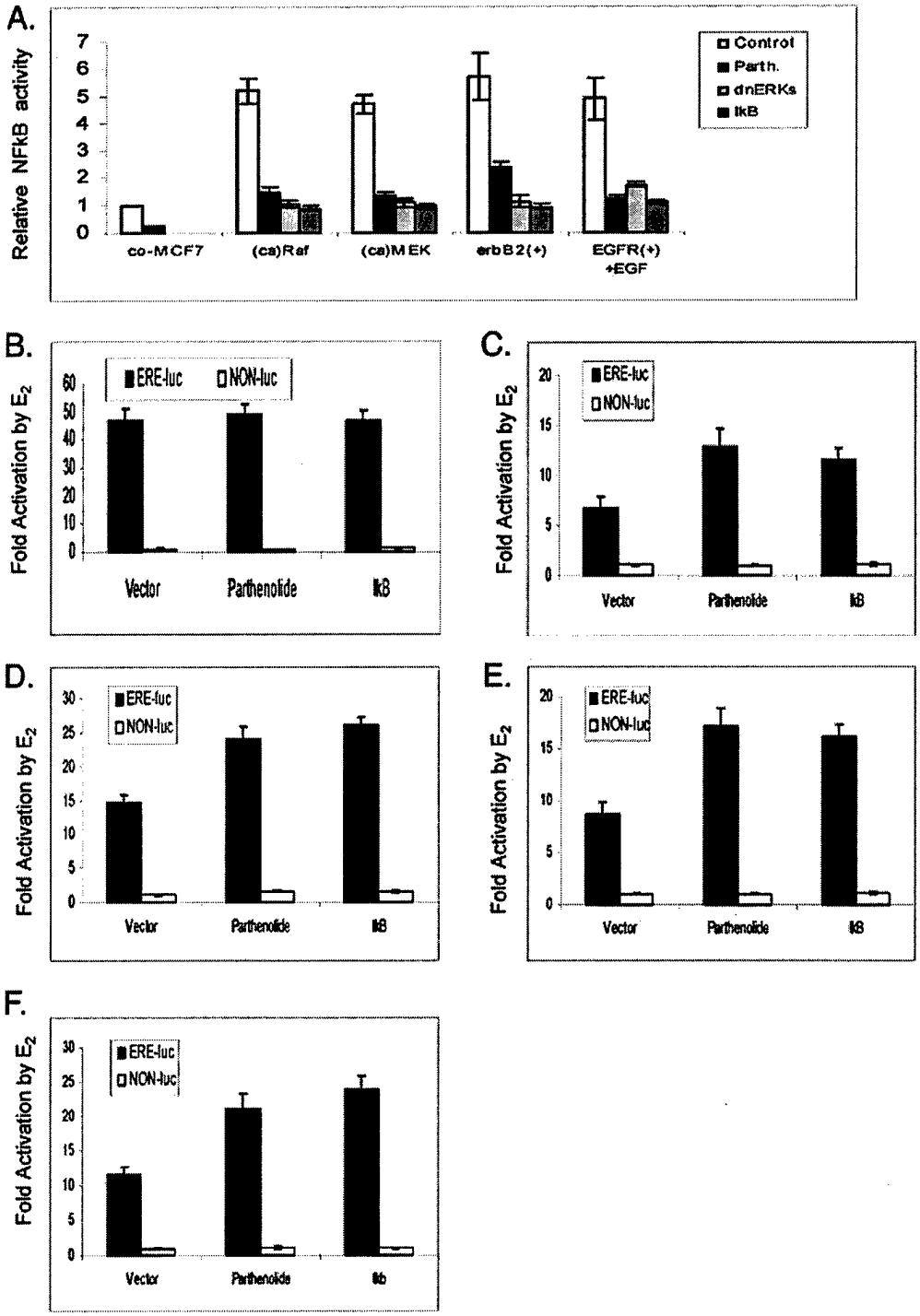

Fig. 5. Elevated NF-κB Activity Is Partially Responsible for ERα Down-Regulation

A, Expression of dnERKs inhibits NF-κB activity. Cells were transiently transfected with 1.25 μg dnERKs, (ca) IκB or vector control DNA and 0.75 μg NF-κB luciferase reporter construct, and basal NF-κB activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative NF-κB activity. The *white bars* represent basal NF-κB-luc activity, the *black bars* represent NF-κB-luc activity in the presence of 2 μM Parthenolide, the *light gray bars* represent NF-κB-luc activity in the presence of dnERKs, and the *dark gray bars* represent NF-κB activity in the presence of (ca)IκB. B–F, co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg vector DNA or (ca)IκB, and 0.75 μg ERE-luc or NON-luc reporter constructs and were treated with control or estrogen-containing media. One set of plates was treated with 2 μM Parthenolide (DMSO at 1:10,000 was used as vehicle control). Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each performed in triplicate. *Error bars* represent SEM.

sured by Elk activation) is elevated relative to co-MCF-7 in our model cell lines with hyperactive MAPK (Fig. 4A). The ERK2Δ19–25 construct greatly reduces this elevated elk activation to levels comparable to co-MCF-7, *i.e.* it greatly reduces nuclear MAPK activity. If a nuclear substrate were responsible for the down-regulation of ERα, then expression of the ERK2Δ19–25 construct in these ERα− models would be expected to block the activation of that substrate by MAPK, and thus lead to the restoration of ERα activity and expression. However, expression of the ERK2Δ19–25 construct did not lead to the restoration of ERα in any of the cell lines (Fig. 4, B–E); in fact, it was unable to increase ERα activity to above basal levels in all cases and was not able to return ERα activity to the level obtained with dnERK transfection. This indicates that a cytoplasmic substrate of MAPK is responsible for the down-regulation of ERα in these breast cancer cell lines.

The Role of NF-κB in ERα Down-Regulation by Hyperactive MAPK

NF-κB activity is elevated in hormone-independent and ER-negative breast tumors (24), and hyperactivation of MAPK leads to enhanced NF-κB activity through induction of autocrine factors such as HB-EGF (29, 30, 51, 52). We therefore asked whether NF-κB activity is elevated in MCF-7 breast cancer cells with elevated MAPK activity. As shown in Fig. 5A, NF-κB activity is about 5-fold higher than parental MCF-7 in all of our model cell lines. This elevated NF-κB activity is attributable to hyperactivation of MAPK because NF-κB activity is returned to normal levels (basal levels in co-MCF7 cells) by dnERKs 1 and 2 (Fig. 5A). The elevated NF-κB activity can also be inhibited by Parthenolide [a specific pharmacological inhibitor of NF-κB (53)] and through cotransfection of (ca)IκB (which acts as a dominant negative for NF-κB, obtained from Upstate Biologicals, Charlottesville, VA). Both (ca)IκB and Parthenolide, at the usual dose of 2 μM, result in decreased NF-κB activity to levels observed in co-MCF7 cells. Neither Parthenolide nor IκB alter MAPK activity (data not shown); nor does inhibition of NF-κB activity alter ERE-luc activity in the co-MCF7 cells (Fig. 5B). Interestingly, inhibition of elevated NF-κB activity in the hyperactive MAPK cell lines with either Parthenolide or (ca)IκB is about 40–50% as effective in restoring ERα activity as are the dnERKs (Fig. 5, C–F). Increasing doses of Parthenolide do result in further inhibition of NF-κB activity in the hyperactive MAPK cell lines (Fig. 6A); however, this further decrease in NF-κB activity does not result in further increases in ERα activity (Fig. 6B), suggesting that the 40–50% effectiveness in restoration of ERα is the maximal effect. Collectively, these data demonstrate that the indirect but specific elevation of NF-κB activity by hyperactive MAPK plays a role in ERα down-regulation in our cell lines, but it is not the only player. Overall, our data suggest that an additional cytoplasmic MAPK substrate(s), along with NF-κB, leads to ERα down-regulation in MCF-7 cells with hyperactive MAPK.

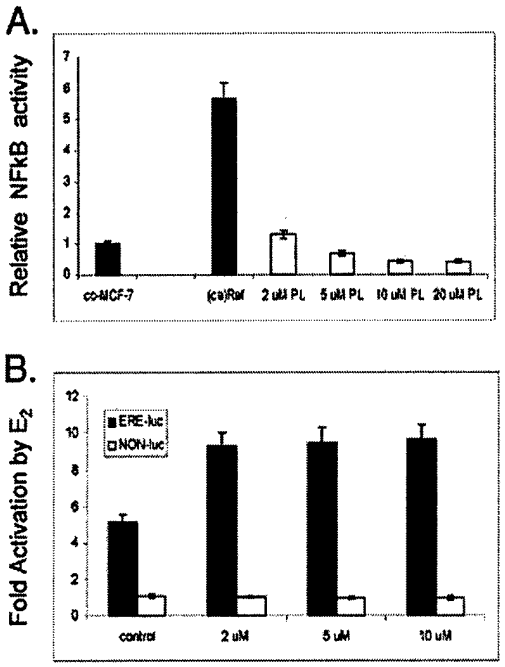

Fig. 6. Elevated NF-κB Activity Has a Threshold Effect on ERα

A, Higher doses of Parthenolide inhibit NF-κB activity to levels below basal MCF-7. (ca)Raf cells were transfected with the NF-κB-luc reporter and luciferase activity was measure in the presence of increasing doses of Parthenolide (2–20 μM). NF-κB activity in co-MCF-7 cells was used for comparison. B, Higher doses of Parthenolide have no further effect on NF-κB activity in cells with hyperactive MAPK. (ca)Raf cells were transiently cotransfected with 1.25μg vector DNA and 0.75 μg ERE-luc or NON-luc reporter constructs and were treated with control or estrogen-containing media. Cells were treated with the indicated doses of Parthenolide (DMSO at 1:10,000 was used as vehicle control). Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each done in triplicate. *Error bars* represent SEM. Similar experiments with the (ca)MEK, erbB-2(+), and EGFR(+) cells yielded similar results (data not shown).

DISCUSSION

The inverse correlation between ERα and EGFR or c-erbB-2 overexpression/ hyperactivation in breast cancer is well established (54–57). Because ERα− tumors display a more aggressive phenotype, have a poor prognosis, and do not respond to antiestrogen therapy, therapeutic targets enabling the restoration of ERα expression may provide beneficial

treatment strategies. Elucidation of the mechanisms involved in the generation of the ERα− phenotype is crucial for the development of such therapies. We have previously demonstrated that hyperactivation of MAPK induced by enhanced expression and/or activation of EGFR or c-erbB-2 results in down-regulation of ERα expression, suggesting that elevated mitogenic signaling induced by overexpression/ hyperactivation of these growth factor receptors is directly responsible for generating the ERα− phenotype (34). Importantly, abrogation of the hyperactive MAPK restores ERα expression and activity in our model cell lines. To identify the MAPK substrate(s) responsible for down-regulation of ERα expression, the specificity of this response to ERK1 *vs.* ERK2 was first examined. It has been reported that although ERK1 and 2 share common substrates, they also display some differential substrate specificity (38). In our previous study, we used pharmacologic inhibitors or dnERK1 and dnERK2 combined to inhibit MAPK activity. To determine whether the inhibition of either ERK alone would restore ERα expression/activity, experiments were performed comparing the ability to restore ERα activity by dnERK1 alone or dnERK2 alone relative to the combination of dnERK1 and 2 (Fig. 1). In these experiments, it did not appear that there was a differential ERK substrate involved in down-regulating ERα. However, it is possible that these constructs may not be completely specific, *i.e.* that dnERK1 may inhibit ERK2 to some extent, and *vice versa*. Therefore, to completely rule out a differential ERK specificity, it would be necessary to knock out expression of each ERK separately using small inhibitory RNA or antisense technology.

We next focused on the role of AP-1 in the MAPK-induced down-regulation of ERα expression. Because ERKs induce the expression of various AP-1 family members (45, 58) and result in elevated AP-1 activity, all of our model cell lines displayed increased AP-1 activity. And although AP-1 has been identified as part of a complex that binds to an enhancer element in the ERα promoter (59), up-regulation of AP-1 activity is seen in ERα− and hormone-independent breast cancers (21, 35, 43, 60). In addition, stable overexpression of c-jun in MCF-7 cells leads to the formation of hormone-independent, ERα− tumors in nude mice (23). Collectively, these data seem to implicate elevated AP-1 activity in ERα down-regulation. However, the data presented here (Fig. 2) represent the first mechanistic study to investigate this correlation, analyzing whether inhibition of elevated AP-1 activity consequent to MAPK hyperactivation can restore ERα expression/activity. Results from these experiments show that inhibition of the elevated AP-1 activity in our cell line models is not enough to restore ERα expression, suggesting that MAPK-induced elevation in AP-1 activity does not, on its own, result in the loss of ERα. This is in apparent contradiction to what might be expected based on the report by Smith *et al.* (23) that overexpression of c-jun leads to the loss of ERα activity and expression in MCF-7 cells. The authors in that report suggest that this might occur either due to squelching of common cofactors required for both ERα-mediated and AP-1-mediated transcriptional activity (similar to that described in Refs. 60 and 61) or maybe through AP-1 activity negatively affecting ERα transcription, although the one AP-1 element identified so far on the ER promoter is a positive regulatory element (59). Because AP-1 molecules can be composed of both homo- and heterodimers, and different dimers have differential affinities for the consensus AP-1 sites, the exact composition of the AP-1 molecule(s) in each instance is likely to influence the final outcome on target genes. It is therefore possible that elevated AP-1 activity due to hyperactive MAPK in our MCF-7-derived cell lines may be due to dimers that markedly differ from those that result from c-jun overexpression, thereby accounting for the very different results reported earlier (23). In addition, AP-1 activity in our hyperactive MAPK cells is three to four times higher than in co-MCF7. Because we don't know the degree to which AP-1 activity is enhanced by c-jun overexpression, it is possible that a threshold level of AP-1 activity is required to alter ERα expression and that this threshold level might be higher than what is seen in our cell lines. Our data here suggests that the negative effects of hyperactive MAPK on ERα transcription are not attributable to MAPK-induced AP-1 but might be due to some other ERK substrate(s), and that inhibiting AP-1 activity is not enough to relieve this effect of hyperactive MAPK.

Data obtained using the ERK2Δ19–25 construct (Fig. 4) confirm the fact that AP-1 does not play a role in MAPK-induced ERα down-regulation, as they indicate that nuclear substrates of MAPK, such as the AP-1 family members, are not responsible for ERα down-regulation. In fact, these data point to a cytoplasmic substrate of MAPK as responsible for ERα down-regulation in our model cell lines. Upon activation, this substrate may itself translocate to the nucleus to affect ERα transcription, or it may activate another molecule, either cytoplasmic or nuclear, which results in the loss of ERα.

We have preliminary data indicating that the down-regulation of ERα occurs via both transcriptional repression and induction of protein degradation (our unpublished data). Although these experiments clearly show that the MAPK effect on ERα is not a direct one in the nucleus, that is, interacting with the ERα promoter to result in transcriptional repression, they do not address what role, if any, MAPK may play in any proteasomal degradation of ERα protein that may be occurring. Several reports indicate that MAPK can directly phosphorylate ERα on serine residue 118 (62, 63), and if the phosphorylation of this residue leads to ubiquitination and degradation, then the abrogation of

nuclear MAPK activity could also eliminate this method of down-regulation. However, this effect cannot be identified in a system with transcriptional repression of ERα smaller posttranslational effects on ERα levels would be masked unless the strong transcriptional repression is relieved. Therefore, to elucidate any role of MAPK hyperactivation in ERα degradation, it would be necessary to use tagged ERα constructs under the control of a heterologous promoter.

RSK1 is the best-characterized cytoplasmic substrate of MAPK. However, it is not the cytoplasmic effector that results in ERα down-regulation (Fig. 3). RSK1 is a kinase that is responsible for the activation of many substrates; among them are multiple transcription factors and several histones (reviewed in Ref. 36). RSK1 was a good candidate as the MAPK substrate responsible for the down-regulation of ERα because we had previously established that a major component of ERα down-regulation in our model cells was transcriptional (our unpublished data). The fact that RSK1 itself alters chromatin structure through the phosphorylation of histones, and that many of its substrates are also involved in transcriptional regulation, led us to investigate it initially. In addition, the data provided by the ERK2Δ19–25 construct further encouraged us to pursue RSK1 because it is a cytoplasmic substrate of MAPK. Also of interest to us was the fact that RSK1 has been reported to phosphorylate ERα directly, on serine reside 167 (37), although in this case it resulted in ligand-independent activation of ER. Although we show that the activation of RSK1 through MAPK does not lead to ligand-independent activation of ERα, as the phosphorylation and results from (37) might suggest, it appears that RSK1 may play a role in the ligand-dependent activation of ERα. Abrogation of signaling through RSK1 did not restore estrogen-induced ER activity, and in fact, it seemed to decrease the ability of estrogen to induce ERα activity, at least in the control (MCF-7) cells. This implicates the phosphorylation of ERα on S167 as potentially important in traditional estrogen-dependent signaling. This result also confirms that the dnRSK1 construct is functional and has an effect on cell signaling. Therefore, a cytoplasmic substrate of MAPK other than RSK1 must be responsible for the down-regulation of ERα induced by hyperactivation of MAPK in these breast cancer cells.

It has been known for some time that NF-κB activity is elevated in hormone-independent and ERα− breast cancers (24). However, the correlation between NF-κB activity and MAPK was less clear—it is now evident that, whereas MAPK does not directly activate NF-κB (either through IκB phosphorylation, or through activation of IKK), hyperactive MAPK enhances NF-κB activity through induction of an autocrine factor, likely HB-EGF (30, 51, 52). Thus, hyperactivation of MAPK can indirectly lead to elevated NF-κB activity. We have shown here that our MCF-7-derived model cell lines all have high basal levels of NF-κB activity as a consequence of ERK1/2 hyperactivation (Fig. 5). Interestingly, inhibiting this level of NF-κB activity (bringing it down to levels normally seen in MCF-7 cells) partially restores ERα activity in these cell lines, indicating that loss of ERα is attributable to elevated NF-κB activity, along with (other) cytoplasmic substrate/s of ERK1/2. It is important to note that, under our experimental conditions, ERE-luc activity mirrors ERα expression [as we have shown earlier (34)], indicating that increase in ERα activity after NF-κB inhibition is attributable to restoration of ERα expression. This is further reinforced by the fact that NF-κB inhibitors have no effect on ERE-luc activity in co-MCF7 cells. Therefore, the enhanced ERE-luc activity in our hyperactive MAPK cell lines after NF-κB inhibition is due to increased ERα expression, rather than due to increased activation of preexisting receptor through modulation of coactivators and/or corepressors. We also find that further reducing NF-κB activity beyond the basal level inherent to MCF-7s does not increase ERα activity any further (Fig. 6), suggesting that down-regulation of ERα due to deregulation of NF-κB activity is a threshold effect. The basal level of activity seen in MCF-7 cells has no adverse effect on ERα; rather, it is the elevation in NF-κB activity beyond this limiting level, which contributes to down-regulating ERα expression. This threshold effect mimics that observed for hyperactivation of MAPK; that is, we had previously observed that neither the basal MAPK activity levels in MCF-7 cells nor the modest elevation of MAPK that occurs in estrogen-independent MCF-7 cells (those long-term adapted for growth in the absence of estrogen such as our co-MCF-7/s cells) has a detrimental effect on ERα expression levels; only hyperactivation to a very high level as seen in ERα− breast cancer cells resulted in down-regulation of ERα expression (34).

This and future studies further elucidating the interaction/s between hyperactive growth factor signaling and ERα expression in breast cancer could have substantial clinical impact in patients. Methylation of the ERα promoter is present at the time of diagnosis of breast cancer in about 25% of ERα− breast cancer cases (64), and in these tumors abrogation of MAPK activity would not be expected to return ERα expression (because demethylation of the ERα promoter would have to occur first). However, in patients with ERα− tumors without any hypermethylation of the promoter, a therapeutic regimen that combines a signal transduction inhibitor/a monoclonal antibody against a key signaling molecule, along with an antiestrogen, could prove beneficial. Iressa, which inhibits the kinase activity of EGFR, and Herceptin, a monoclonal antibody raised against c-erbB-2, can both be expected to reduce MAPK activity, and would be good candidates for such combination therapies. Although further stud-

ies are essential to show that these inhibitors of mitogenic signaling can restore ERα expression/function in breast tumors, coupling signal transduction inhibitors or monoclonal antibodies with antiestrogens could result in therapies that are better tolerated than standard chemotherapy for the treatment of ERα− breast cancer. In addition, once the MAPK substrate/s responsible for ERα down-regulation is identified, that could provide an additional drug target. The implication of elevated NF-κB activity in development of the ERα− phenotype also has clinical potential. High NF-κB activity is thought to contribute to resistance to chemotherapy and to radiation-induced apoptosis in cancers generally (25–27), as well as to influence tumor growth and metastasis through promoting cell survival and cell cycle entry (27, 65). Interestingly, it has been reported that cotreatment with NF-κB inhibitors enhances the paclitaxel sensitivity of MDA-MB-231 breast cancer cells (which have very high basal NF-κB activity) (66). Therefore, combinations of NF-κB inhibitors with standard chemotherapy/radiation might prove generally beneficial in breast and other cancers. Added to this, our data (establishing a role for NF-κB in the loss of ERα expression) make NF-κB a very attractive candidate for combination therapies in breast cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

All cells with hyperactive MAPK were maintained in phenol red-free improved MEM (IMEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% charcoal-stripped calf serum (CCS). Control transfected MCF-7 cells were maintained in IMEM supplemented with 10% fetal bovine serum. All cell lines used in this study have been described earlier (34); the (ca)MEK cells were generated by transfection of MCF-7 cells as described before (34, 39) using a constitutively active MEK construct [a generous gift from Dr. Natalie Ahn (40)]. After transfection, cells were grown in PRF-IMEM/10% CCS supplemented with 10 μg/ml gentamicin (Invitrogen Life Technologies). Hormone treatments after transfections were performed by supplementing the gentamicin containing CCS media with 17β-estradiol (Sigma, St. Louis, MO), at a final concentration of 10^{-9} M; in the case of EGF-treated EGFR(+) cells, the medium was also supplemented with 10 ng/ml EGF (Upstate Biologicals). Parthenolide was obtained from Sigma, and stored as a 10,000× stock in dimethylsulfoxide (DMSO) at −20 C. Lipofectamine and PLUS reagent were obtained from Invitrogen. Cells were seeded in 75-cm^2 T-flasks (Costar, Cambridge, MA) and grown in a forced air humidified incubator at an atmosphere of 5% CO_2 and 37 C.

Plasmids

Luciferase reporter plasmids (pGLB-mERE and pGLB-mNON) were obtained by the insertion of an altered MMTV promoter containing a tandem repeat of a consensus ERE or a scrambled version of the same sequence instead of the glucocorticoid response element into the *Hin*dIII site of the pGLB basic plasmid (Promega Corp., Madison, WI) (67). The dnERK1 and dnERK2 constructs and parental vector pCep4L were kindly provided by Dr. Melanie Cobb (University of Texas Southwestern, Dallas, TX). The TAM67 construct was a gift of Dr. Powel Brown (Baylor College of Medicine Breast Center, Houston, TX). The ERK2Δ19–25 construct was a gift of Dr. Michael Weber (University of Virginia, Charlottesville, VA), and Dr. John Blenis (Harvard Medical School, Boston, MA) kindly provided the dnRSK1 and pRK7 (parental vector) constructs. pCep4L (dnERK parental vector), pCDNA3 (TAM67 and ERK2Δ19–25 parental vector), and pRK7 (dnRSK1 parental vector) were used as empty vector controls in transient transfections. The Stratagene PathDetect kit was the source of the AP-1-luc, NF-κB-luc, pFAelk, and pFRluciferase plasmids. The constitutively active IκB plasmid [(ca)IκB or IκB (S32A/S36A) in pUSE] was obtained from Upstate Biologicals.

Transfections

Cells were plated at approximately 60–70% confluence in Falcon six-well plates and allowed to attach overnight. They were then transfected using the LipoPlus method. Briefly, 100 μl serum-free IMEM per well was mixed with 4 μl Plus reagent and 2 μg total plasmid DNA [0.75 μg of each luciferase containing reporter plasmid, and 1.25 μg total of the effector plasmid(s)]. This mixture was incubated at room temperature for 15 min, combined with a mixture containing 100 μl serum-free IMEM and 4 μl Lipofectamine, and then incubated for an additional 15 min. This mixture was then added to the cells, which were incubated for 3 h at 5% CO_2 and 37 C. After transfection, the cells were washed with PBS and incubated for 48 h in appropriate treatment media. Control transfection with a cytomegalovirus (CMV)-luc plasmid was performed in all experiments as a measure of transfection efficiency. All transfections in each experiment were performed in triplicate.

Luciferase Assays

Cells were washed twice with PBS and lysed in 200 μl of passive lysis buffer (Promega) with gentle agitation for 15 min at room temperature. Ten to 25 μl of lysate (actual volumes determined based on the amount needed to get CMV-luc readings in the middle of the linear range of the instrument) were used to measure luciferase activity using Promega's Luciferase Assay system. The luciferase values (relative light units) were normalized for protein concentration. Values were corrected by the subtraction of relative light units per microgram of protein for the mock-transfected cells. Triplicates were averaged, and fold activation by estrogen was plotted; the *error bars* represent SEM. Results shown are representative of at least three experiments with similar results, each done in triplicate.

Ligand Binding Assays

Whole cell extracts were prepared from cells grown to 75–80% confluence as described before (34). Extracts were diluted to a concentration of 2 mg/ml, and incubated with 10 nM [^{3}H]-17β-estradiol in the presence and absence of ×100 unlabeled estradiol for 16 h at 4 C. Dextran-coated charcoal was then used to adsorb free hormone and pelleted by centrifugation. Aliquots of the supernatant were counted in 10 ml of liquid scintillation fluid using a Beckman Coulter Inc. (Fullerton, CA) liquid scintillation counter. Receptor expression values were determined as described in Ref. 68 and are expressed as femtomoles per milligram of protein. Assays were performed a minimum of three times on each cell line over time in culture.

Acknowledgments

We acknowledge Drs. Sofia Merajver, Francis Kern, and James Rae for critical review of this manuscript and thoughtful and helpful discussions.

Received February 5, 2004. Accepted March 22, 2004.

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This work was supported by Department of Defense Grant DAMD17-01-0247 (to J.N.H.); American Cancer Society Grant RSG-01-0330-01 (to D.E.A.); Susan G. Komen Foundation Grant BCTR2000565 (to D.E.A.); and National Institutes of Health-University of Michigan Cancer Center Support Grant 5 P30 CA46592.

J.N.H. and S.M. made equal contributions.

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